AD_____

Award Number: W81XWH-09-1-0128

TITLE: Imaging CXCL12-CXCR4 Signaling and Inhibition in Ovarian Cancer

PRINCIPAL INVESTIGATOR: Gary D. Luker, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
                           Ann Arbor, MI 48109-1274

REPORT DATE: Annual

TYPE OF REPORT: July 2012

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2012	Annual	1 July 2011 – 30 June 2012

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Imaging CXCL12-CXCR4 Signaling and Inhibition in Ovarian Cancer	5b. GRANT NUMBER: W81XWH-09-1-0128
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Gary D. Luker	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
E-Mail: gluker@umich.edu	

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
Ann Arbor, MI 48109-1274

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

CXCR4 and its chemokine ligand CXCL12 may represent new molecular targets for chemotherapy in patients with ovarian cancer. CXCR4 is expressed on ovarian cancer cells in approximately 50% of patients, and expression of this receptor correlates with poor prognosis. High levels of CXCL12 are present in ascites of patients with ovarian cancer, providing a local source of chemokine ligand in the tumor microenvironment. CXCL12 signaling through CXCR4 activates pathways that could promote tumor growth, dissemination, and resistance to chemotherapy. However, our knowledge of how these signaling pathways function in the tumor microenvironment of ovarian cancer in vivo is poorly understood. We propose to develop new molecular imaging technologies and signaling reporters to analyze CXCR4 signaling in vivo and determine to what extent inhibiting CXCL12-CXCR4 signaling produces disease regression in mouse models of ovarian cancer.

15. SUBJECT TERMS
Optical imaging, chemokine, chemokine receptor, signal transduction

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			
U	U	U	UU	6	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Year 3 Annual Report: W81XWH-09-1-0128
Imaging CXCL12-CXCR4 Signaling and Inhibition in Ovarian Cancer

1. Introduction. CXCR4 and its chemokine ligand CXCL12 are potential targets for molecular therapy of ovarian cancer. Receptor CXCR4 is expressed by ovarian cancer cells in approximately 50% of patients. High levels of CXCL12 are present in ascites of patients with ovarian cancer, providing a local source of chemokine ligand in the tumor microenvironment. CXCL12 signaling through CXCR4 activates pathways that could promote tumor growth, invasion, metastasis, and resistance to chemotherapy. To develop CXCL12-CXCR4 as molecular targets for chemotherapy in ovarian cancer, there is an unmet clinical need to understand how these signaling pathways function in the tumor microenvironment of ovarian cancer *in vivo*. We propose to develop new molecular imaging technologies and signaling reporters to analyze CXCR4 signaling *in vivo* and determine to what extent inhibiting CXCL12-CXCR4 signaling produces disease regression in mouse models of ovarian cancer.

2. Body.
Aim 1. To construct ovarian cancer cell lines with molecular imaging reporters to quantify activation of CXCR4 and downstream effector molecules.
A. Time period: Months 1-16
B. Tasks to be completed:
1. Establish ovarian cancer cell lines (Hey-8, Skov3i.p.1) stably expressing CXCR4 imaging reporters for receptor homodimerization, recruitment of β-arrestin 2, and activation of AKT.
2. Test responses of each reporter cell line to stimulation with CXCL12 and inhibition with CXCR4 inhibitors AMD3100 and TF1403 in cell-based assays.
3. Test CXCL12-dependent activation of signaling pathways in reporter cells using biochemical assays including Western blotting.

Progress. 1. We established Hey-8 cell lines with these reporters except for the AKT construct. We designed and tested different reporter constructs for AKT activity, but none generated specific signals for this pathway in cell based assays. Performance of our imaging reporters in terms of signal to background was notably better for Hey-8 cells, so our experiments in cell culture and animals were accomplished with this ovarian cancer cell line.
2 – 3. We previously reported validation of Hey-8 reporter cell lines with AMD3100, the only clinically approved inhibitor of CXCL12-CXCR4 binding and signaling. We tested kinetics of inhibition using a reporter for CXCR4 interaction with β-arrestin 2. Treatment of Hey-8 reporter cells with CXCL12 rapidly (within 40 minutes) produced bioluminescence above basal levels, and this effect was blocked completely with AMD3100 (Fig 1). These data establish the capability of this reporter to quantify acute changes in CXCL12-CXCR4 activation or inhibition.

Aim 2. Image and quantify activation of CXCR4 signaling and pharmacodynamics of CXCR4 inhibitors in mouse models of ovarian cancer.
A. Time period: Months 13-36
B. Tasks to be completed:
1. Establish intraperitoneal ovarian cancer xenografts of Hey-8 or Skov3i.p. cells stably expressing CXCR4 imaging reporters. Xenografts will be established in $Ncr^{nu/nu}$ (nude) mice.

2. Perform bioluminescence imaging and MRI studies of CXCR4 signaling and tumor progression.

3. Quantify inhibition of CXCR4 signaling with known and candidate CXCR4 inhibitors and establish to what extent inhibition of CXCR4 signaling correlates with tumor growth and overall survival.

Progress. 1. We previously described successful generation of ovarian cancer xenografts with Hey-8 cells expressing CXCR4 imaging reporters. For reasons described above, we have not been able to accomplish imaging studies with Skov3i.p. cells.

2-3. We used the CXCR4-β-arrestin 2 reporter to quantify inhibition of CXCR4 in living mice and test effects of CXCL12 to reduce cytotoxicity of the chemotherapeutic drug cisplatin against Hey-8. In cell culture studies, CXCL12 minimally affected cell death produced by increasing concentrations of cisplatin (Fig 2). In a mouse xenograft model, treatment with AMD3100 inhibited CXCR4 signaling, as evidenced by a decrease in complementation bioluminescence from association of CXCR4 with β-arrestin 2 (Fig 3). Treatment with cisplatin plus AMD3100 did not reduce CXCR4 signaling to a greater extent than AMD3100 alone. As decreased in our previous report, we used fluorescence from far red fluorescent protein FP650 to quantify tumor burden rather than MRI because of problems with respiratory motion artifacts on MRI. We quantified survival of mice implanted with Hey-8 CXCR4-β-arrestin 2 reporter cells and Hey-8 cells secreting CXCL12 in response to the following treatments: 1) vehicle alone; 2) cisplatin (4 mg/kg every 5 days intraperitoneal); 3) AMD3100 (25 mg/ml delivered by 2 week osmotic infusion pumps); or 4) both cisplatin and AMD3100. Treatment began one week after implanting tumor cells and continued for two weeks, the maximum period of delivery for the AMD3100 osmotic infusion pumps. We discontinued treatment with cisplatin after removing AMD3100 or control osmotic infusion pumps. There was a trend toward improved survival in mice treated with both cisplatin and AMD3100, although differences from cisplatin alone were not significant (Fig 4). We hypothesize that continuing therapy throughout the full period of the experiment could make these differences significant. Such a treatment protocol likely is more feasible in patients for whom more advanced medical monitoring and supportive care is available than can be provided for mice.

Key Research Accomplishments.
- Established that AMD3100 blocks CXCL12-dependent recruitment of β-arrestin 2 during extended periods of therapy in a mouse xenograft model.
- Found a trend toward improved survival of mice treated with combination therapy of AMD3100 to block CXCL12-CXCR4 and the standard chemotherapeutic drug cisplatin.

Reportable Outcomes.
- Publication: Ray P, Lewin SA, Mihalko LA, Schmidt BT, Luker KE, Luker GD. Noninvasive imaging reveals inhibition of ovarian cancer by targeting CXCL12-CXCR4. Neoplasia 2011; 13(12): 1152-1161.

Conclusion.
Our research established imaging reporters for CXCL12 binding to CXCR4 and activation of downstream signaling molecules. Using these reporters, we established the ability to monitor activation and inhibition of CXCR4 signaling in real time in cell-based assays and

living mice. We also established that treatment with AMD3100 extended survival of mice with Hey-8 ovarian cancer xenografts, as published in Neoplasia.

Our research demonstrated a trend toward improved survival in tumor-bearing mice treated with cisplatin, a standard chemotherapeutic drug for ovarian cancer, and AMD3100. This trend was evident even though mice were treated with these drugs for only two weeks, which represents less than one-third of the total observation period. The limited duration of treatment is due to the challenges of administering multi-agent chemotherapy to mice and managing toxicities of therapy. With more advanced methods of medical monitoring and support available to human patients with ovarian cancer, it may be feasible to continue combination therapy over longer periods of time and establish significant improvements in survival.
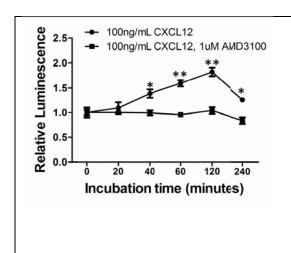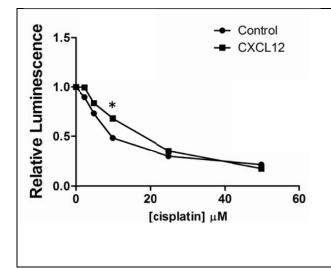
Supporting Data:

Aim 2.

Figure 1. AMD3100 rapidly inhibits CXCR4-β-arrestin 2 complementation signal. Hey-8 cells expressing a luciferase complementation reporter for CXCR4 interaction with β-arrestin 2 were incubated with 100 ng/ml CXCL12 in the presence of an inhibitory concentration (1 µM) of AMD3100 or vehicle control. Graph shows mean values ± SEM for induction of bioluminescence above basal values for cells not treated with CXCL12 (n = 4 per condition). CXCL12 rapidly activates reporter bioluminescence, which is inhibited completely by AMD3100.

Figure 2. CXCL12 minimally reduces cytotoxicity of cisplatin to Hey-8 ovarian cancer cells. Hey-8 cells expressing a bioluminescent reporter for cell viability were co-cultured with Hey-8 cells that secrete CXCL12 (CXCL12) or control Hey-8 cells that do not secrete this chemokine (control). Cells were incubated for 2 days with increasing concentrations of cisplatin, a standard chemotherapeutic drug used in ovarian cancer. The graph shows mean values for bioluminescence with error bars smaller than the symbols (n = 10 per condition). *, $p < 0.05$.